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# Development of Enzyme-Linked Immunosorbent Assays for Residue Analysis of Diflubenzuron and BAY SIR 8514

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16. ABSTRACT

Three enzyme-linked immunosorbent assays (ELISA's) were developed for the benzoylphenylurea insect growth regulators (IGRs) diflubenzuron, BAY SIR 8514, and some of their analogues. All three ELISA's were based on antibodies raised against an *N*-carboxypropyl hapten of diflubenzuron while a diflubenzuron phenylacetic acid derivative coupled to a different carrier than the immunizing antigen was used as the coating antigen. One ELISA was sensitive for diflubenzuron, BAY SIR 8514, and closely related IGRs while a second assay was sensitive for diflubenzuron while demonstrating minimal cross-reactivity with BAY SIR 8514. In a third case, rabbit anti-diflubenzuron IgG was coupled to alkaline phosphatase, thus eliminating one step in the classical ELISA. None of these assays demonstrated significant cross-reactivity with the benzamide, urea, phenylurea, or aniline components of diflubenzuron or BAY SIR 8514. Each of the three assays was shown to be as sensitive as the recommended HPLC methodology for the analysis of diflubenzuron in water. Low cost was found to be a major advantage of these solid-phase immunoassays over classical procedures.

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# Development of Enzyme-Linked Immunosorbent Assays for Residue Analysis of Diflubenzuron and BAY SIR 8514

Siong I. Wie and Bruce D. Hammock*

Three enzyme-linked immunosorbent assays (ELISA's) were developed for the benzoylphenylurea insect growth regulators (IGRs) diflubenzuron, BAY SIR 8514, and some of their analogues. All three ELISA's were based on antibodies raised against an *N*-carboxypropyl hapten of diflubenzuron while a diflubenzuron phenylacetic acid derivative coupled to a different carrier than the immunizing antigen was used as the coating antigen. One ELISA was sensitive for diflubenzuron, BAY SIR 8514, and closely related IGRs while a second assay was sensitive for diflubenzuron while demonstrating minimal cross-reactivity with BAY SIR 8514. In a third case, rabbit anti-diflubenzuron IgG was coupled to alkaline phosphatase, thus eliminating one step in the classical ELISA. None of these assays demonstrated significant cross-reactivity with the benzamide, urea, phenylurea, or aniline components of diflubenzuron or BAY SIR 8514. Each of the three assays was shown to be as sensitive as the recommended HPLC methodology for the analysis of diflubenzuron in water. Low cost was found to be a major advantage of these solid-phase immunoassays over classical procedures.

In the preceding paper (Wie et al., 1982), the synthesis of haptens for the insect growth regulators (IGRs) diflubenzuron [Dimilin, *N*-[[(4-chlorophenyl)amino]-carbonyl]-2,6-difluorobenzamide], BAY SIR 8514 [*N*-[[[4-(trifluoromethoxy)phenyl]amino]carbonyl]-2-chlorobenzamide], and their protein conjugates were described, and high titers of specific antibodies to diflubenzuron were produced in rabbits. In the present study enzyme-linked immunosorbent assays (ELISA's) for both diflubenzuron and BAY SIR 8514 were developed and shown to be highly specific and of adequate sensitivity for residue work. Immunoassays are physical (in contrast to biological) assays based upon the principle of competitive binding. Although the terminology used to describe the assays is intimidating, they are easy to perform and highly reproducible. This report is part of a larger study designed to examine the potential of immunochemical technology for solving specific problems in environmental chemistry.

## MATERIALS AND METHODS

**Reagents.** Alkaline phosphatase conjugated goat anti-rabbit IgG was prepared as described by Engvall and Perlmann (1972) and also obtained from Miles Laboratory (Elkhart, IN). Ovalbumin (OA), keyhole limpet hemocyanin (KLH), and bovine serum albumin (BSA) were purchased from Calbiochem (San Diego, CA), *p*-nitrophenyl phosphate and Sepharose 4B were from Sigma Chemical Co. (St. Louis, MO), isobutyl chloroformate, *tert*-butylamine, and cyanogen bromide were from Eastman Kodak Co. (Rochester, NY), 1-aminophenylacetic acid and diethanolamine were from Aldrich Chemical Co. (Milwaukee, WI), and goat anti-rabbit immunoglobulin was obtained from Antibodies Incorporated (Davis, CA).

**Antisera.** The anti-diflubenzuron antibodies were obtained from New Zealand white rabbits as described in the preceding paper (Wie et al., 1982). Thus, sera A and B were obtained from rabbits immunized with *N*-(carboxypropyl)diflubenzuron–KLH prepared by the active ester method (Figure 1). The other antigens were perpared by the water-soluble carbodiimide method and rabbits were inoculated as follows: rabbits C and D [*N*-(carboxypropyl)diflubenzuron–BSA], rabbit E [*N*-(carboxypropyl)diflubenzuron–KLH], and rabbits F and G (diflubenzuron phenoxyacetate–KLH). Sera used in this study were collected 7 months after immunization although usable titers were obtained much earlier.

**Preparation of Coating Antigens by the Mixed Anhydride Method.** The haptens, diflubenzuron phenylacetic acid or *N*-(carboxypropyl)diflubenzuron (0.052 mmol), were dissolved in 500 μL of dry dioxane. To this solution was added tri-*n*-butylamine (0.062 mmol) and then isobutyl chloroformate (0.062 mmol). The resulting mixture was stirred at room temperature for 30 min and then added dropwise to a solution of ovalbumin (150 mg) or KLH (100 mg) in 15 mL of 0.2 M borate buffer, pH 8.7, and stirred at room temperature overnight. The conjugates were then dialyzed extensively in 7 mM phosphate buffer containing 0.15 M NaCl and 0.02% $NaN_3$. This procedure resulted in the covalent binding of 2.5 mol of hapten/50 kilodaltons of protein.

**Immunosorbent.** Diflubenzuron phenylacetate–OA was coupled to CNBr-activated Sepharose 4B at a concentration of 1 mg of protein/mL of Sepharose 4B (Cuatrecasas, 1970). The immunosorbent was washed on a Büchner funnel with $Na_2CO_3$ solution, $H_2O$, and 0.2 M glycine–HCl buffer, pH 2.8, before equilibration with phosphate-buffered saline (PBS; 7 mM $PO_4^{2-}$, pH 7.7, and 0.15 M NaCl). The anti-diflubenzuron *N*-carboxypropyl sera (rabbits C or D) were then passed through the immunosorbent (7 mL). The initial effluent and a PBS wash were combined and concentrated by negative pressure to the initial volume and dialyzed against PBS at pH 7.7. The absorbed protein was eluted with 10 mL of 0.2 M glycine–HCl buffer at pH 2.8. The pH of the eluted material was adjusted to 7.7 by the addition of NaOH, and subsequently it was concentrated and dialyzed against PBS at pH 7.7.

**Enzyme-Linked Immunosorbent Assay (ELISA).** ELISA was carried out according to Voller et al. (1976a,b, 1979) with slight modifications. A summary of the ELISA procedure is given in Figure 2. All the reactions were performed in polystyrene plates (Dynatech or Gilford cuvettes). A volume of 200 μL was used in the Dynatech plates, whereas 300 μL was employed for Gilford plates throughout the study. The coating antigens were diluted in 0.1 M sodium carbonate buffer, pH 9.6, containing 0.02% $NaN_3$ and added to each well. The plates were then kept overnight at 4 °C. The coated plates were washed 3 times with PBS containing 0.05% Tween 20 and 0.02% $NaN_3$ (PBS–Tween) (step 2, Figure 2). After addition of diluted antiserum in PBS–Tween, plates were incubated for 2 h at room temperature (step 3). After three further

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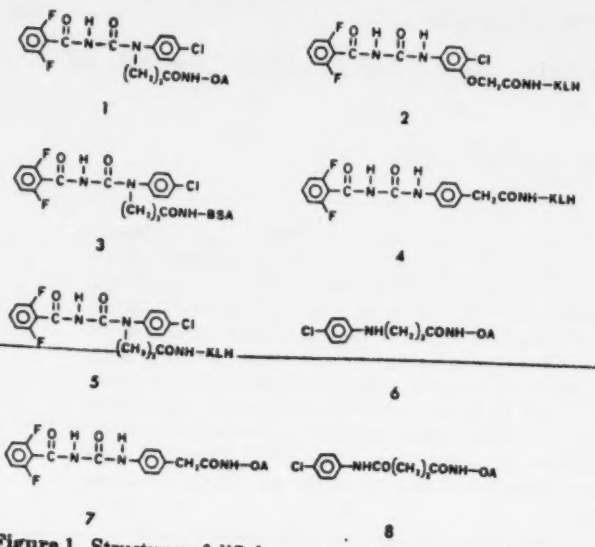



**Figure 1.** Structures of diflubenzuron and hapten protein conjugates. *N*-(Carboxypropyl)diflubenzuron–OA (1), diflubenzuron phenoxyacetate–KLH (2), *N*-(carboxypropyl)diflubenzuron–BSA (3), and *N*-(carboxypropyl)diflubenzuron–KLH (5) were used as immunizing antigens, while diflubenzuron phenylacetate–KLH (4), *N*-(carboxypropyl)-4-chloroaniline–OA (6), diflubenzuron phenylacetate–OA (7), and 4-chloroaniline hemisuccinamide–OA (8) were used as coating antigens in ELISA's.

washes with PBS–Tween, the goat anti-rabbit Ig–alkaline phosphatase conjugate diluted in PBS–Tween (1:2500) was added and allowed to incubate at room temperature for 2 h (step 4). After another three washes, phosphatase activity was measured following the addition of a 1 mg/mL solution of *p*-nitrophenyl phosphate in 10% (w/v) diethanolamine buffer, pH 9.8. The reaction was stopped 30–40 min later by addition of 75 μL of 3 N NaOH, and the color intensity was measured in a microcell read in a Zeiss spectrophotometer at 405 nm in some early work or in a Gilford EIA reader also at 405 nm (step 5).

The preparation of inhibition curves was as follows. One milliliter of appropriately diluted anti-benzoylphenylurea serum was transferred to each of several 13 × 100 mm culture tubes (step 1, Figure 2). To these antibody solutions was added 20 μL of hapten (i.e., diflubenzuron) dissolved in acetonitrile or dioxane with increasing hapten concentrations. After incubation at room temperature overnight, the serum was added to the hapten-coated plates. The assays were then performed as described above. Maximum binding and background binding were assessed by adding solvent only or no antibody, respectively, to the system.

In some cases a series of known diflubenzuron concentrations were added to either water taken from a nearby storm sewer, WHO synthetic water, or whole or low fat milk. To 0.9 mL of this material was added enough of a concentrated antibody solution in 10 × PBS–Tween to give

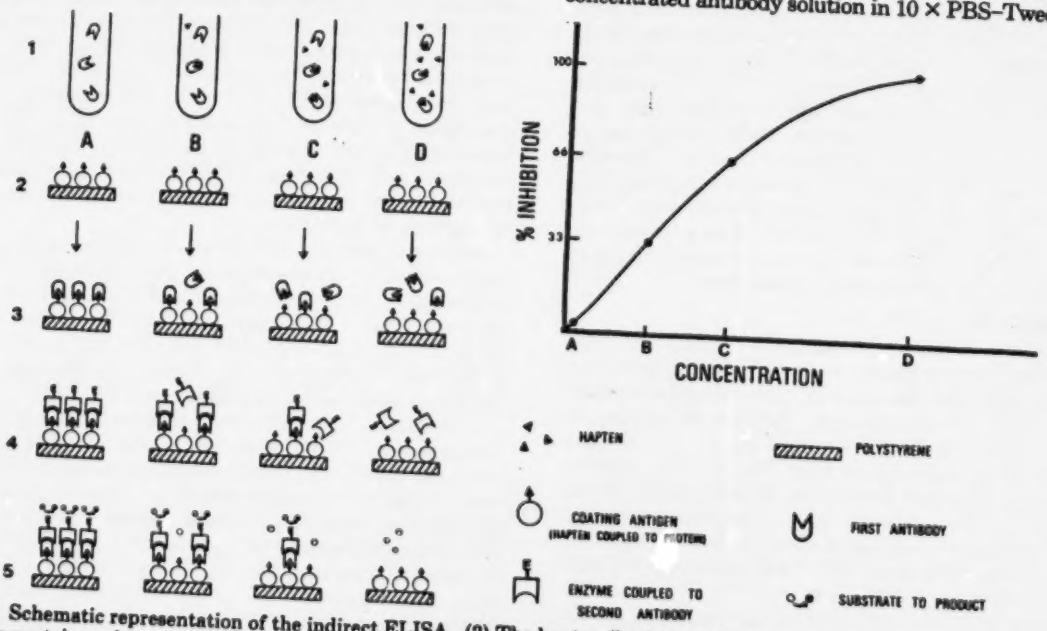



**Figure 2.** Schematic representation of the indirect ELISA. (2) The hapten (in this case diflubenzuron phenylacetate) is covalently linked to a protein such as ovalbumin to give the coating antigens. The coating antigen is then irreversibly bound to the surface of the polystyrene cuvette. The inclusion of surfactant in subsequent washes prevents further protein binding. (3) The contents of tubes (top) containing a constant amount of first antibody (in this case the rabbit anti-diflubenzuron *N*-carboxypropyl derivative) are added to a series of cuvettes previously incubated as above with a constant amount of coating antigen. As increasing amounts of diflubenzuron are added to the tubes (B–D), less and less of the first antibody is free to bind to the coating antigen. All of the first antibody not bound to the coating antigen is washed out of the cuvette while that bound to the coating antigen cannot be washed out. (4) A second antibody is then added (in this case goat anti-rabbit antibody) covalently coupled to an enzyme such as alkaline phosphatase. The amount of second antibody bound to the plate and thus the amount of alkaline phosphatase bound are dependent upon the amount of the first antibody bound. Excess enzyme conjugated second antibody is then washed out. (5) Finally substrate is added. In this case the amount of *p*-nitrophenol produced is directly proportional to the amount of first antibody bound to the plate and thus is inversely proportional to the amount of diflubenzuron in the knowns and unknowns (A–D). The maximum yellow color is observed with tube A. As increasing amounts of diflubenzuron are added, the development of color is inhibited, leading to a standard curve as shown on the right. The assay was in some cases modified by the covalent linking of alkaline phosphatase directly to the first antibody, thus eliminating one step in the assay. The assay is either performed in a disposable cuvette pack or a microtiter plate so that 50–96 operations can be carried out simultaneously. For further descriptions of the ELISA procedure, see Voller et al. (1976a,b, 1979) and Hammock and Mumma (1980).

Table I. Serum Titers of Anti-diflubenzuron Antibodies Determined by Enzyme-Linked Immunosorbent Assay (ELISA)[a]

| rabbit | immunizing antigen | coating antigen: diflubenzuron phenylacetate–OA | coating antigen: *N*-(carboxypropyl)-diflubenzuron–OA | coating antigen: *N*-(carboxypropyl)-*p*-chloroaniline–OA[b] |
|---|---|---|---|---|
| A | *N*-(carboxypropyl)diflubenzuron–KLH | 400 | 25 600 | <100 |
| B | *N*-(carboxypropyl)diflubenzuron–KLH | 100 | 25 600 | <100 |
| C | *N*-(carboxypropyl)diflubenzuron–BSA | 6400 | 25 600 | <100 |
| D | *N*-(carboxypropyl)diflubenzuron–BSA | 6400 | 25 600 | <100 |
| E | *N*-(carboxypropyl)diflubenzuron–KLH | 1600 | 25 600 | <100 |
| F | diflubenzuron phenoxyacetate–KLH | 400 | 400 | <100 |
| G | diflubenzuron phenoxyacetate–KLH | 400 | 400 | <100 |
| NRS | | <100 | <100 | <100 |

[a] Dynatech plates were coated with a 5 μg/mL solution of the indicated antigen. Serial dilutions of antiserum were then added to the plates and the assay was carried out as described under Materials and Methods. The reported titer was determined from the highest dilution of serum which yielded an $OD_{405nm}$ greater than 0.3. [b] Titers of <100 were also observed for all sera when the *p*-chloroaniline *N*-succinamide–OA conjugate was used as the coating agent.

the normally used antibody dilution in 1 mL. The assay was then carried out as described above and compared with a standard curve for diflubenzuron as normally determined after correction for a 10% dilution.

In the case of the modified indirect ELISA, rabbit anti-diflubenzuron IgG conjugated with alkaline phosphatase was used in place of rabbit anti-diflubenzuron antiserum. After this coupled reagent was added to the washed plate, the substrate (1 mg/mL solution of *p*-nitrophenyl phosphate) was added, and after incubation at room temperature for 120 min, the absorbence was measured at 405 nm (see the legend to Figure 2).

All assays were run in triplicate, and the standard deviation for all points of the inhibition curves (within run variation) were ≤5% of the optical density observed. The coefficient variation for the percent inhibition for any particular dose of one inhibitor was ≤3.5% of the actual value obtained from at least three runs performed on widely separate occasions.

## RESULTS

Initial experiments using *N*-(carboxypropyl)diflubenzuron–OA (Figure 1) as the coating antigen in ELISA's were unsuccessful in inhibition studies with diflubenzuron and BAY SIR 8514 because of the strong binding between the antibody and coating antigen. Subsequently, diflubenzuron phenylacetate–OA was employed as the coating antigen and was found useful in inhibition experiments. The binding of the antisera with two different coating antigens in the ELISA test is shown in Table I. All sera gave strong binding to the *N*-(carboxypropyl)diflubenzuron coating antigen except sera F and G, which were obtained from rabbits immunized with diflubenzuron phenoxyacetate–KLH. The binding of these antibodies to diflubenzuron phenylacetate–OA coated cuvettes was also significant, and their titers were consistent with the results obtained in radioimmunoassay as described in the companion paper (Wie et al., 1982). Hence, sera from rabbits C and D exhibited strong affinity to both [$^{14}$C]diflubenzuron and diflubenzuron phenylacetate–OA, and they were used in the following experiments. All sera demonstrated very high titer (>25 000) to their corresponding immunizing carriers (BSA or KLH) as determined by ELISA. The specificity of this reaction was further demonstrated first by using normal rabbit serum in place of immune sera and then by using the carbofuran phenol–OA conjugate prepared by the mixed anhydride method as the coating antigen in the ELISA procedure. Titers lower than 100 were observed for the sera in both cases.

After the initial checkerboard titration of both antigen and antisera (Voller et al., 1976a,b), the optimal concentration for diflubenzuron phenylacetate–OA conjugate was determined as shown in Figure 3. Positive reactions were

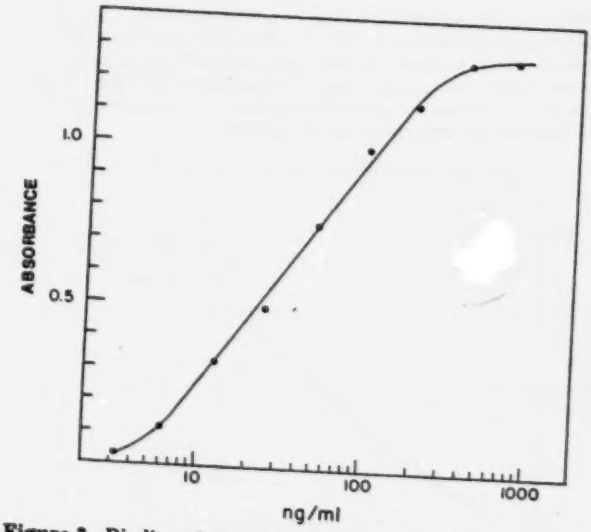



**Figure 3.** Binding of a constant amount of antiserum (rabbit C, 1:2200 dilution) to an increasing concentration of diflubenzuron phenylacetate–OA conjugates used to coat the ELISA plate. Subsequently, goat anti-rabbit Ig conjugated with alkaline phosphatase was added, and the enzymatic activity was demonstrated by the addition of a 1% solution of *p*-nitrophenyl phosphate.

detected for both sera with the concentration of coating antigen as low as 60 ng/mL, and the plateau was reached at concentrations greater than 4 μg/mL for the antiserum concentration used. Subsequently, a solution of 5 μg/mL diflubenzuron phenylacetate–OA as the coating antigen was used throughout the study.

Inhibition studies were then carried out to investigate the ability of various haptens to prevent the binding of anti-diflubenzuron antibodies (rabbit C) to the diflubenzuron phenylacetate–OA coated plates. The results shown in Figure 4 are expressed as percent inhibition of a standard amount of antibody plotted against a variable concentration of hapten. Diflubenzuron exhibited strong inhibition with the 50% inhibition point ($I_{50}$) at a concentration of 48 ng/mL, and ultimately, 90% inhibition could be obtained. This inhibition curve is linear over a wide range of hapten concentrations, thus allowing between 8 and 200 ng/mL diflubenzuron to be readily quantitated without prior dilution or concentration.

Competition displayed by BAY SIR 8514 is also shown in Figure 3 and indicated an $I_{50}$ at 120 ng/mL. However, it could not replace all the antibody combining sites as a maximum level of 68% inhibition was obtained. On the other hand, both 2,6-difluorobenzamide and the succinic

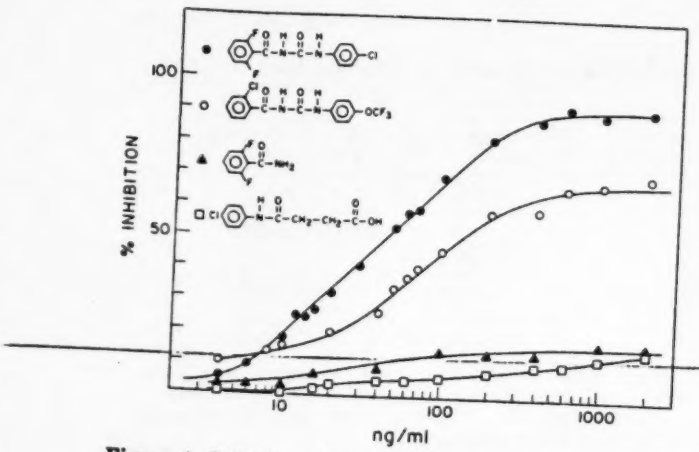



**Figure 4.** Inhibition of binding of anti-*N*-(carboxypropyl)diflubenzuron antibodies (rabbit C, 1:2200 dilution) by various haptens in an indirect ELISA using diflubenzuron phenylacetate–OA as the coating antigen. Diflubenzuron (●), BAY SIR 8514 (○), 2,6-difluorobenzamide (▲), and 4-chloroaniline hemisuccinamide (□) are shown as competitors.

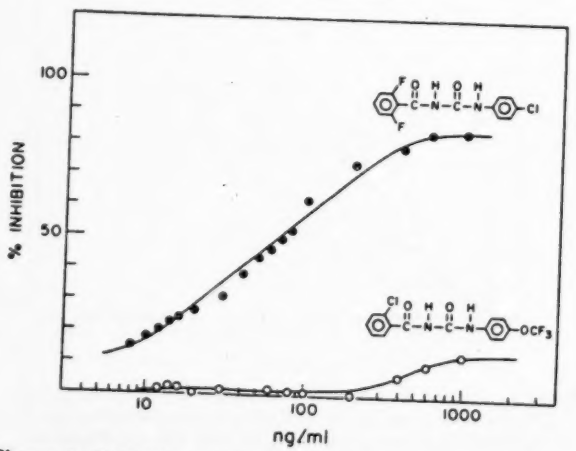



**Figure 5.** Inhibition of binding of anti-*N*-(carboxypropyl)diflubenzuron antibodies (rabbit D, 1:2200 dilution) by two haptens in an indirect ELISA using diflubenzuron phenylacetate–OA as the coating antigen.

monoamide of *p*-chloroaniline were shown to be poor inhibitors. These results indicate that (a) the anti-*N*-(carboxypropyl)diflubenzuron antibodies strongly recognized the benzamide portion of the molecule and (b) the existence of the urea moiety and phenyl ring also played a critical role for the better complementary fix of this hapten to the antibody combining sites. Rabbit D, which was also immunized with *N*-(carboxypropyl)diflubenzuron–BSA, yielded a serum that was highly specific for diflubenzuron in the ELISA (Figure 5). This serum exhibited much less cross-reaction than that from rabbit C with BAY SIR 8514 and other closely related haptens. The $I_{50}$ for diflubenzuron was at a concentration of 67 ng/mL. Therefore, one is able to distinguish the two IGRs with the two sera described. The ability of immunochemical methods to differentiate structurally similar compounds at the residue level is clearly demonstrated by these results and could obviously be further enhanced by affinity chromatography of the antisera. It was interesting to note that addition of diflubenzuron in dioxane rather than in acetonitrile resulted in an even greater ability of the resulting ELISA to distinguish diflubenzuron and BAY SIR 8514. This

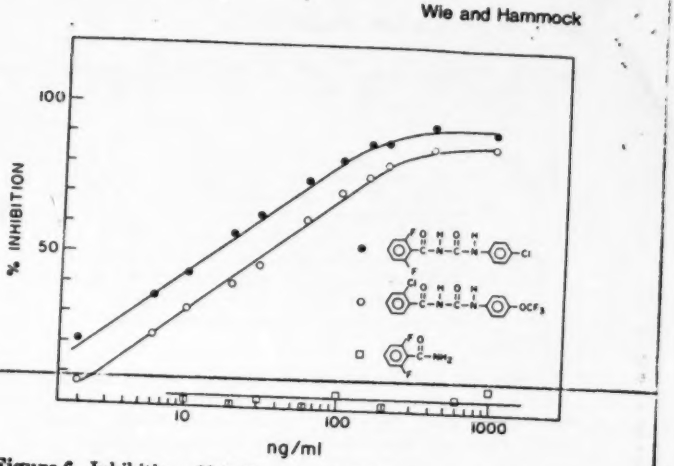



**Figure 6.** Inhibition of binding of anti-diflubenzuron (rabbit C, 1:200) by various haptens in a modified indirect ELISA in which the anti-diflubenzuron IgG was conjugated with alkaline phosphatase.

possibly resulted from dioxane reducing some less specific hydrophobic interaction common to both benzoylphenylureas.

Inhibition studies were also performed by using other haptens (Table II). For rabbit C, all the benzylphenylurea compounds (1–12) gave significant inhibition while all the N-substituted compounds for both diflubenzuron (7, 8) and BAY SIR 8514 (9, 10) exhibited better competition than their parent compounds. Typical inhibition curves are shown in Figure 6 where diflubenzuron *N*-propargyl and diflubenzuron *N*-(ethoxycarbonyl)methyl derivatives were employed as haptens. The $I_{50}$'s were shown to be 0.64 and 0.74 ng/mL, respectively. Moreover, these two compounds displayed much steeper inhibition curves, and the maximum inhibition was obtained at a lower concentration of <5 ng/mL. This result implies that the modified diflubenzuron or BAY SIR 8514 with a tertiary nitrogen atom containing the extra side chain was more strongly recognized by the antibody than its coating antigen. The result could be anticipated since these compounds more closely resemble the injected hapten than the parent compounds and other derivatives. Such compounds are very unlikely to be encountered in environmental analyses of diflubenzuron or BAY SIR 8514, but these assays could be very sensitive for the alkyl-substituted aniline derivatives of diflubenzuron examined by Bull and Ivie (1980). It is apparent that highly sensitive solid-phase immunoassays for small molecules can be achieved if appropriate combinations of antiserum and antigen are chosen. The minor metabolites, 2′-hydroxydiflubenzuron and 3′-diflubenzuron (Table II, compounds 11 and 12) were shown to be strong inhibitors with the 2′-hydroxydiflubenzuron exhibiting better binding than its 3′-hydroxy counterpart. Such compounds can be readily removed from diflubenzuron by partition or chromatographic techniques. On the other hand, other closely related compounds (13–20) were found to be poor inhibitors as were other commonly used pesticides (21–24).

The results from rabbit D were similar to those obtained from rabbit C except that antibodies from rabbit D recognized poorly the haptens of BAY SIR 8514 and other closely related compounds such as penfluron (2) and PH 60-43 (4). However, N-substituted BAY SIR 8514 derivatives (9, 10) also exhibited strong affinity to the antibody because of their close resemblance to the immunizing antigen. This antiserum also demonstrated the specificity toward the benzoylphenylureas seen in rabbit C.

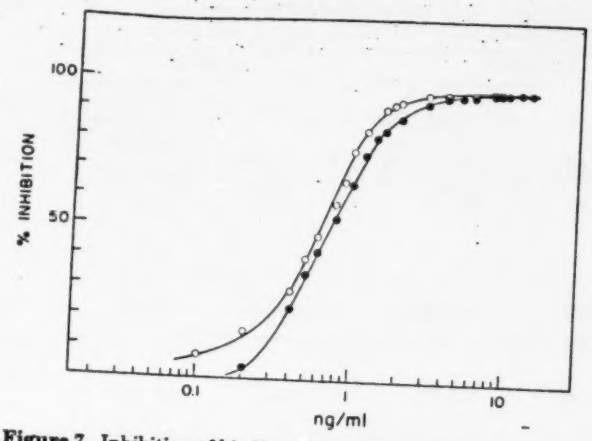



**Figure 7.** Inhibition of binding of anti-diflubenzuron (rabbit C, 1:2200) by the diflubenzuron *N*-propargyl (○) and diflubenzuron *N*-(ethoxycarbonyl)methyl derivative (●) in an indirect ELISA.

In order to simplify the ELISA for quantitation of diflubenzuron, experiments were carried out to couple alkaline phosphatase to the rabbit anti-diflubenzuron (rabbit C) and thus eliminate an antibody–antigen reaction in the ELISA procedure. A ratio of 1:2 (w/w) for the enzyme and immunoglobulin was used in the preparation of this conjugate, and a concentration of 1 μg/mL of immunoglobulin was employed for the experiment. As shown in Figure 7, the enzyme-conjugated antibodies retain their binding capacity, and their use affords a sensitivity similar to that of the two step ELISA (Figure 5). This particular assay gave $I_{50}$'s of 15 and 37 ng/mL for diflubenzuron and BAY SIR 8514, respectively. In addition, it also exhibited a specificity similar to that observed in the previous experiments (Table II, Figure 4). For instance, 2,6-difluorobenzamide did not bind to the antibody–alkaline phosphatase conjugate.

When stagnant water in which mosquitoes were breeding or World Health Organization synthetic water was used in the ELISA, there was no significant interference (at the 5% level) with the sensitivity, slope, or reproducibility of the assay, and no cleanup was required prior to analysis. Thus, the linear region for diflubenzuron extended from below 10 to above 200 ppb with the indirect assay. Somewhat greater sensitivity (1 ppb) could be obtained with the modified indirect assay (Figure 6). When skim milk or homogenized milk was fortified with diflubenzuron in acetonitrile solution, interference was observed, and the assay sensitivity was 4 times lower than when distilled water was used instead of milk. Therefore, the results obtained from the ELISA determination for diflubenzuron in milk must be multiplied by a factor to correct for the difference due to interference when this combination of antibody and coating antigen are used. Alternatively, the milk could be diluted 4× before analysis to remove interference, resulting in a linear region for diflubenzuron determination extending from below 40 to above 800 ppb in milk. Interestingly, equal interference was obtained with both skim milk and whole milk, indicating that protein rather than lipid interfered with the assay when 900 μL of milk was used. This assay interference could be largely overcome by increasing the serum concentration used from 2200 to 1400×, further supporting the hypothesis that nonspecific protein binding contributed to the interference.

If needed, the assay sensitivity could be readily increased by simply substituting methylumbelliferyl phosphate (which yields a fluorescent product) for the *p*-nitrophenyl phosphate used as the substrate in these studies (Voller et al., 1976a,b, 1979), using a commercially available, radiolabeled second antibody, performing the initial incubations at low temperature to decrease the apparent $K_d$ of the antibody, or using nonequilibrium conditions. Interference also could be reduced while increasing specificity and sensitivity by simply performing one or more of the cleanup steps of the classical HPLC procedures, although extensive cleanup would defeat, in part, the purpose of the assay in reducing the cost of residue analysis.

## DISCUSSION

Since the benzoylphenylurea compounds, diflubenzuron and BAY SIR 8514, are projected to be used as commercial insecticides, the fate of these compounds in the environment is exceedingly important. The classical methods of diflubenzuron analysis include GLC or HPLC. Since extensive cleanup procedures are needed, these methods are very expensive and tedious (Corley et al., 1974; DeMilo et al., 1978; DiPrima et al., 1978; Lawrence and Sundaram, 1976; Maini and Deseo, 1976; Oehler and Holman, 1975; Schaefer and Dupras, 1977; Worobey and Webster, 1977, 1978; Rabenort et al., 1978; Monem and Mumma, 1981) (Table III). For these reasons, simple and sensitive assay techniques are needed in order to analyze large numbers of samples. Immunoassay has been widely used in the health and biological science (Yalow, 1978), and its application to environmental contaminants is being explored (Hammock and Mumma, 1980). The major step in developing immunoassays for the residue analysis of pesticides as in the case of the benzoylphenylureas was the development of suitable antisera. By use of an antigen in which protein was attached to the center of the diflubenzuron molecule [in *N*-(carboxypropyl)diflubenzuron–BSA conjugates], specific anti-diflubenzuron antibodies from rabbits were obtained as described in Wie et al. (1982). Although RIA using [$^{14}$C]diflubenzuron as a marker was shown to be useful in the detection of anti-diflubenzuron antibodies, its use in analysis was found to be impractical due to the low specific activity of the [$^{14}$C]diflubenzuron available. Rather than prepare a high specific activity diflubenzuron radiolabel, an alternative method, ELISA, was then explored since it has been shown that the amount of either antibody or antigen determined by the ELISA method correlates well with values obtained by radioimmunoassay. Because of the sensitivity, ease of measurement, good stability, and long shelf life, enzymes have proven to be good markers in nonisotopic immunoassays. In addition, the ELISA method is simple to carry out and determinations require only a few hours. With no automation over 30–50 ELISA samples can be easily run per man day in this laboratory while automated devices enhance this productivity 10–100-fold. Although the procedures outlined in Figure 2 appear tedious, it should be recalled that the operations are carried out simultaneously on a large number of samples.

Attempted inhibition with diflubenzuron in ELISA's using the *N*-(carboxypropyl)diflubenzuron–OA conjugate as coating antigen was not successful because diflubenzuron could not compete with the strong preference binding of the antibody to the *N*-(carboxypropyl)diflubenzuron coating antigen. However, diflubenzuron phenylacetate–OA was demonstrated to be useful as a coating antigen, and the specific assays for both diflubenzuron and BAY SIR 8514 are shown in Figures 4 and 5. Such results are commonly encountered in immunoassay and illustrate an approach by which the sensitivity and specificity of ELISA's can be varied while using a single antibody. As shown in Table I the sera raised against *N*-(carboxypropyl)diflubenzuron–KLH or –BSA had very high titers when

Table II. $I_{50}$ and Maximal Inhibition Caused by Several Compounds in Standard ELISA's Using Diflubenzuron Phenylacetate-OA as Coating Antigen and Two Sera Raised against *N*-Carboxypropyl Derivative of Diflubenzuron[a]

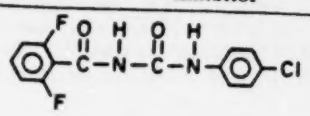
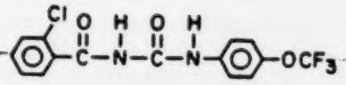
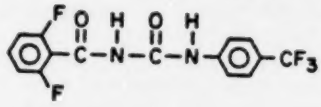
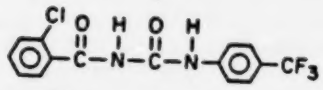
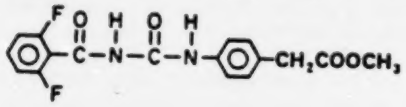
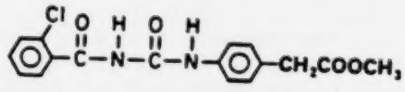
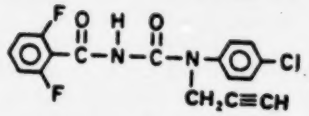
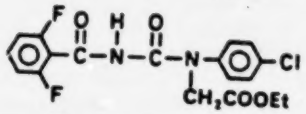
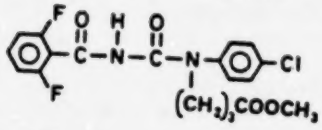
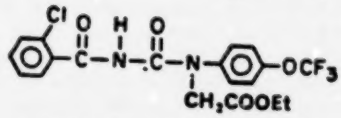
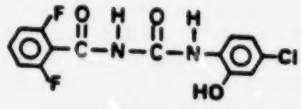
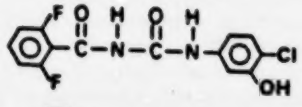
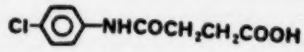
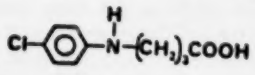
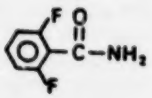
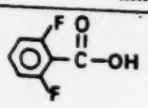
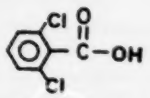
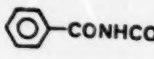
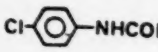
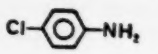
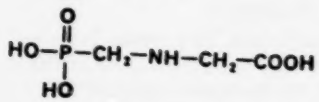
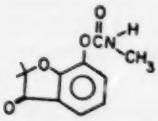
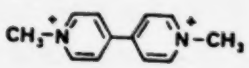
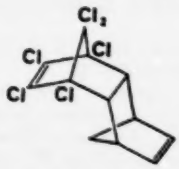
| | | rabbit C | | rabbit D | |
|---|---|---|---|---|---|
| no. | inhibitor | $I_{50}$, ng/mL | max % inhibition by large amt, μg/mL | $I_{50}$, ng/mL | max % inhibition by large amt, μg/mL |
| 1 |  | 48 | 90 (2) | 65 | 84 (1) |
| 2 |  | 120 | 68 (2) | >1000 | 14 (1) |
| 3 |  | 190 | 77 (2) | 630 | 64 (2) |
| 4 |  | 220 | 65 (1) | >2000 | 33 (2) |
| 5 |  | 11 | 96 (0.1) | 92 | 96 (2) |
| 6 |  | 16 | 95 (0.1) | 360 | 89 (2) |
| 7 |  | 0.64 | 98 (0.1) | 1.4 | 98 (0.1) |
| 8 |  | 0.74 | 97 (0.1) | 1.7 | 96 (0.1) |
| 9 |  | 0.43 | 98 (0.1) | 2.7 | 98 (1) |
| 10 |  | 8.8 | 95 (0.1) | 13.5 | 96 (2) |
| 11 |  | 18 | 90 (2) | 220 | 80 (2) |
| 12 |  | 63 | 95 (2) | 76 | 95 (2) |
| 13 |  | >2000 | 15 (2) | >3500 | 7 (3.5) |
| 14 |  | >2000 | 10 (2) | >3500 | 8 (3.5) |
| 15 |  | >5000 | 20 (5) | >3500 | 32 (3.5) |

Table II (*Continued*)

| no. | inhibitor | rabbit C $I_{50}$, ng/mL | rabbit C max % inhibition by large amt, μg/mL | rabbit D $I_{50}$, ng/mL | rabbit D max % inhibition by large amt, μg/mL |
|---|---|---|---|---|---|
| 16 |  | >10000 | 5.8 (10) | ND[b] | ND[b] |
| 17 |  | >8000 | 0 (8) | >8000 | 0 (8) |
| 18 |  | >4000 | 0 (4) | >4000 | 0 (4) |
| 19 |  | >10000 | 10.3 (10) | ND[b] | ND[b] |
| 20 |  | >2000 | 15 (2) | >3500 | 4 (3.5) |
| 21 |  | >32000 | 0 (32) | >32000 | 0 (32) |
| 22 |  | >8000 | 10 (8) | >3500 | 4 (3.5) |
| 23 |  | >50000 | 0 (50) | >50000 | 0 (50) |
| 24 |  | >50000 | 0 (50) | >50000 | 0 (50) |

[a] Gilford cuvettes were coated with a 5 μg/mL solution of diflubenzuron phenylacetate–OA. The serum dilutions for rabbit C and rabbit D were 1:2200 and 1:2000, respectively. Increasing concentrations of inhibitors were added to the antibody solution and the assay was performed as described under Materials and Methods. [b] Not determined.

*N*-(carboxypropyl)diflubenzuron–OA was used as the coating antigen. However, the very high affinity of the antibodies for the coating antigen led to very low assay sensitivity for diflubenzuron. By use of diflubenzuron phenylacetate–OA as the coating antigen, a significantly lower titer was observed since the assay now selected a subpopulation of antibodies which recognized the diflubenzuron phenylacetate. As shown in Table II, the affinity of diflubenzuron (1) for the antibody population selected in this ELISA approaches (rabbit C) or exceeds (rabbit D) the affinity for the phenylacetate (5), resulting in a sensitive assay. When the antibody population has a higher affinity for the compound to be analyzed than for the coating antigen, very high sensitivities can be obtained as illustrated for those compounds with a trisubstituted nitrogen (7–10).

Two hapten to protein ratios (molar ratio 15:1 and 30:1) were employed in the preparation of coating antigens for diflubenzuron phenylacetate–OA. It was found that there was no significant difference between the two preparations in terms of their properties as the coating antigen and in the detection and quantitation of haptens in ELISA procedures. Diflubenzuron phenylacetate–KLH was also used successfully as the coating antigen; however, a higher background was also observed, which is commonly the case when KLH is used as a coating antigen.

In some cases immunoassays can be improved by passing the serum through an affinity chromatography column. There are two major applications of such a procedure. first, an unwanted antibody population can be removed by passing the serum through a column to which the antigen is attached. For instance, if an immunoassay was to be used for the analysis of diflubenzuron in bovine blood, the antiserum could be passed through a column to which BSA was attached. In other cases the desired antibody can be absorbed to an antigen covalently linked to a solid matrix and then selectively eluted, giving an enriched antibody population as was done in this study.

The antibodies that recognized diflubenzuron phenylacetate were shown to be a unique population in the serum since they could be removed specifically by immunosorbent, namely, diflubenzuron phenylacetate–OA covalently attached to solid-phase Sepharose 4B. Serum passing through this immunosorbent was deprived of the activity in binding to diflubenzuron phenylacetate–OA coated plates, while the anti-*N*-(carboxypropyl)diflubenzuron titer was not significantly affected. Moreover, antibodies (rabbit D) subsequently eluted from the immunosorbent retained

Table III. Comparison of Classical (HPLC and GLC) and Immunochemical Methods for the Analysis of Diflubenzuron Residues in Water and Milk

| method | substrate | sample size, mL | cleanup steps prior to analytical procedure | assays per man-day[b] | total time for analysis of one bank of samples | approximate cost of specialized equipment, $ | approximate reagent cost per sample,[c] $ | limit of sensitivity,[d] ppb | recovery of spiked sample | reproducibility |
|---|---|---|---|---|---|---|---|---|---|---|
| Thompson-Hayward[a] 1A (HPLC) | stagnant water | 250 | 5 | 5 | 8 h | 12 000 | 4.00 | 10.0 | 86 | ±6 |
| modified indirect ELISA | stagnant water | 0.5 | 0 | >50 | 4 h | 1 800 | 0.20 | 1.0 | 100 | ±3.5 |
| Thompson-Hayward[a] 15B (EC-GLC) | whole milk | 20 | 19 | 0.5 | 5 days | 10 000 | 11.00 | 50.0 | 90 | ±8 |
| indirect ELISA | whole milk | 0.2 | 0 | >50 | 6 h | 1 800 | 0.20 | 40.0 | 100 | ±3.5 |

[a] Recovery and reproducibility data were provided by Thompson-Hayward Chemical Co. Recoveries of spiked samples were lower in our hands. The estimate of assays per man-day and the total time for the analysis of one bank of samples was taken after several months work on analysis of benzoylphenylureas carried out at the University of California Mosquito Control Research Laboratory, Fresno, CA. See Literature Cited for detailed descriptions of the HPLC- and GLC-based methods. [b] The major expense in any analytical procedure is the worker time involved, which can be estimated from the assays run per man-day. The speed of the immunochemical procedures can be dramatically increased by the use of semiautomated or automated equipment. [c] This figure only includes the cost of reagents. All disposable materials are used in the ELISA assay and their cost is included in this estimate. This estimate does not include the time spent in cleaning or the cost of purchasing the glassware used in the classical procedure. [d] For the classical procedure, the limit of sensitivity is taken as that level of a compound which gives a peak detectable from base-line noise with 5% confidence. For the immunoassay procedures, the linear region of the inhibition curve delineates assay sensitivity. The statistical limit of sensitivity is lower.

their binding for diflubenzuron phenylacetate, and they were used successfully for determination of diflubenzuron in the ELISA with slightly improved sensitivity (linear region 5–200 ppb) in the standard indirect assay.

In many fields immunoassay is the method of choice. It is thus surprising that it has found such limited application in environmental chemistry (Ercegovich, 1976). Recent studies on aflatoxins (Sun and Chu, 1977; Pestka et al. 1981), *S*-bioallethrin (Wing et al., 1978; Wing and Hammock, 1979), paraquat (Fatori and Hunter, 1980), benzimidazoles (Newsome and Shields, 1981), parathion (Ercegovich et al., 1981), and other compounds demonstrate the potential of immunochemical technology for the analysis of new and pressing problems in environmental chemistry. For certain compounds we have found immunoassays to be significantly faster and cheaper as well as more sensitive and reproducible than classical procedures. These advantages are summarized in Table III for the analysis of diflubenzuron. Immunochemical technology should certainly be used to supplement classical residue procedures, especially for compounds which do not lend themselves to ready analysis by classical means.

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